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(54) Title: HYBRID WITH INTERFERON- α AND AN IMMUNOGLOBULIN Fc LINKED THROUGH A NON-IMMUNOGENIC PEPTIDE (57) Abstract Disclosed is a hybrid recombinant protein consisting of human interferon, preferably interferon- α (IFN α), and human immunoglobulin Fc fragment, preferably γ 4 chain, joined by a peptide linker comprising the sequence Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser (SEQ ID NO:1).		

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Hybrid with Interferon- α and an Immunoglobulin Fc Linked through a Non-Immunogenic Peptide

5 Background of the invention

Interferon- α ("IFN α ") was among the first of the cytokines to be produced by recombinant DNA technology and has been shown to have therapeutic value in conditions such as inflammatory, viral, and malignant diseases. Several IFN α preparations, including those purified from the natural
10 sources and those generated by recombinant DNA technology, have been used or are being tested in a variety of malignant and viral diseases. IFN α can cause regression of some established tumors and induce positive responses in some viral infections. So far, IFN α has been approved or tested in many countries for indications such as: Kaposi's sarcoma; hairy cell leukemia; malignant melanoma; basal cell carcinoma; multiple myeloma; renal cell carcinoma, hepatitis B; hepatitis C; venereal warts, Herpes I/II,
15 varicella/herpes zoster; and mycosis fungoides.

Most cytokines, including IFN α , have relatively short circulation half-lives since they are produced *in vivo* to act locally and transiently. The serum half-life of IFN α is only about two to eight hours (Roche Labs. Referon A, Schering Intron A, *Physicians' Desk Reference*, 47th edition, 1993, pp. 2006-2008, 2194-2201). To use IFN α as an effective systemic therapeutic, one needs relatively large
20 doses and frequent administrations. For example, one of the recommended therapeutic strategies for the AIDS-related Kaposi's sarcoma starts with an induction dose of 36 million IU daily for 10 to 12 weeks, administered as an intramuscular or subcutaneous injection, followed by a maintenance dose of 36 million IU, three times a week. (Roche Labs. Referon A, *Physicians' Desk Reference*, 47th edition, 1993, pp. 2006-2008). Such frequent parenteral administrations are inconvenient and painful.
25 Further, toxic effects, which are probably caused by the high dosage, are a problem for certain

patients. Skin, neurologic, endocrine, and immune toxicity have been reported. To overcome these disadvantages, one can modify the molecule to increase its circulation half-life or change the drug's formulation to extend its release time. The dosage and administration frequency can then be reduced while increasing the efficacy. It was reported that doses of less than nine million units had been well tolerated, while doses more than 36 million units can induce severe toxicity and significantly alter patient status. (Quesada, J.R. et al., *J. Clin. Oncol.*, 4:234-43, 1986). It is possible to decrease substantially the toxic effects by producing a new form IFN α which is more stable in the circulation and requires smaller doses. Efforts have been made to create a recombinant IFN α -gelatin conjugate with an extended retention time (Tabata, Y. et al., *Cancer Res.* 51:5532-8, 1991). A lipid-based encapsulated IFN α formulation has also been tested in animals and achieved an extended release of the protein in the peritoneum (Bonetti, A. and Kim, S. *Cancer Chemother Pharmacol.* 33:258-261, 1993).

Immunoglobulins of IgG and IgM class are among the most abundant proteins in the human blood. They circulate with half-lives ranging from several days to 21 days. IgG has been found to increase the half-lives of several ligand binding proteins (receptors) when used to form recombinant hybrids, including the soluble CD4 molecule, LHR, and IFN- γ receptor (Mordenti J. et al., *Nature*, 337:525-31, 1989; Capon, D.J. and Lasky, L.A., U.S. Patent number 5,116,964; Kurschmer, C. et al., *J. Immunol.* 149:4096-4100, 1992). However, such hybrids can present problems in that the peptide at the C-terminal of the active moiety and the peptide at the N-terminal of the Fc portion at the fusion point creates a new peptide sequence, which is a neoantigen, and which can be immunogenic. The invention relates to a IFN α -Fc hybrid which is designed to overcome this problem and extend the half-life of the IFN α .

Summary of the invention

The present invention relates to a hybrid recombinant protein which consists of two subunits. Each subunit includes a human interferon, preferably $\text{IFN}\alpha$, joined by a peptide linker which is primarily composed of a T cell inert sequence, linked to a human immunoglobulin Fc fragment, preferably the $\gamma 4$ chain. The $\gamma 4$ chain is preferred over the $\gamma 1$ chain because the former has little or no complement activating ability.

The C-terminal end of the $\text{IFN}\alpha$ is linked to the N-terminal end of the Fc fragment. An additional $\text{IFN}\alpha$ (or other cytokine) can attach to the N-terminal end of any other unbound Fc chains in the Fc fragment, resulting in a homodimer for the $\gamma 4$ chain. If the Fc fragment selected is another chain, such as the μ chain, then, because the Fc fragments form pentamers with ten possible binding sites, this results in a molecule with interferon or other cytokine linked at each of ten binding sites.

The two moieties of the hybrid are linked through a T cell immunologically inert peptide (*e.g.*, Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser (SEQ ID NO:1)). This peptide itself is immunologically inactive. The insertion of this peptide at the fusion point eliminates the neoantigenicity created by the joining of the two peptide moieties. The linker peptide also increases the flexibility of these moieties and allows retention of the biological activity. This relatively long linker peptide helps overcome the possible steric hindrance from the Fc portion of the hybrid, which could interfere with the activity of the hybrid.

The hybrid has a much longer half-life than the native $\text{IFN}\alpha$. Due to the linker, it is also designed to reduce the possibility of generating a new immunogenic epitope (a neoantigen) at what would otherwise be the fusion point of the $\text{IFN}\alpha$ and the immunoglobulin Fc segment.

Cytokines are generally small proteins with relatively short half-lives which dissipate rapidly

among various tissues, including at undesired sites. It is believed that small quantities of some cytokines can cross the blood-brain barrier and enter the central nervous system, thereby causing severe neurological toxicity. The IFN α linked to Fc γ of the present invention would be especially suitable for treating hepatitis B or C, because these products will have a long retention time in the vasculature (upon intravenous administration) and will not penetrate undesired sites.

The specific hybrid described can also serve as a model for the design and construction of other cytokine-Fc hybrids. The same or a similar linker could be used in order to reduce the possibility of generating a new immunogenic epitope while allowing retention of the biological activity. Cytokine-Fc hybrids in which interleukin-2 is the cytokine, or hybrids including other cytokines, could be made using the same techniques.

Detailed Description of Making and Using the Invention

The hybrid molecule of the invention includes an interferon moiety linked through a unique linker to an immunoglobulin Fc moiety. Preferably, the C-terminal ends of two interferon moieties are separately attached to each of the two N-terminal ends of a heavy chain γ 4 Fc fragment, resulting in a homodimer structure. A unique linker peptide, Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser (SEQ ID NO:1), was created to link the two moieties. The complete nucleotide sequence of the preferred γ 4 hybrid (including the linker and the Fc moiety) appears in SEQ ID NO: 7. The linker is located at amino acid residue numbers 189 to 204.

The advantage of the hybrid over the native cytokine is that the half-life *in vivo* is much longer. The hybrid including interferon and the γ 4 chain Fc homodimer is larger than the native interferon. Because the pores in the blood vessels of the liver are large, this larger molecule is more suitable for use in treating hepatitis, where the virus responsible primarily affects the liver.

The linker peptide is designed to increase the flexibility of the two moieties and thus maintain their biological activity. Although the interferon and the immunoglobulin are both of human origin, there is always a possibility of generating a new immunogenic epitope at the fusion point of the two molecules. Therefore, the other advantage of the linker of the invention, which consists mainly of a T cell inert sequence, is to reduce immunogenicity at the fusion point. Referring to SEQ ID NO:7, it can be seen that if the linker (residue numbers 189-204) was not present, a new sequence consisting of the residues immediately before number 189 and immediately after 204 would be created. This new sequence would be a neoantigen for the human body.

Human IFN α is derived from a family of several different genes. More than 24 species have been identified so far, from gene and protein sequence data. They differ from each other by anywhere from a few to a maximum of 35 amino acids. Most of the species have a signal peptide sequence of 23 amino acid residues and a mature amino acid sequence of 166 amino acid residues (Goeddel, D.V. et al., *Nature*, 290:20-26, 1981; Weissmann, C. and Weber, H., *Prog. Nuc. Acid Res. Mol. Biol.* 33:251-300, 1986; Zoon, K.C., *Interferon*, 9:1-12, 1987).

IFN α 2 (also called IFN α A) is one of the most intensively studied interferon species. The recombinant version of IFN α 2 has been used as a therapeutic for several years. Two IFN α 2 recombinant products, IFN α 2a and IFN α 2b, are now commercially available. They differ only in one amino acid at position 23, and there is no significant difference in biological activity between them (von Gabain, A., et al., *Eur. J. Biochem.* 190:257-61, 1990).

IFN α 2a was selected as the fusion partner for the interferon hybrid of the invention, although the IFN α 2b or any other interferon species (including IFN β) can be used as well. It is also possible to make similar constructs with other cytokines, such as interleukin-1 or interleukin-2. The same linker

could be used, or another one which is not immunogenic and which maintains the biological activity of the construct could be substituted.

The advantages of the $\gamma 4$ chain as the Fc moiety in the hybrid is that it is stable in the human circulation. The $\gamma 4$ chain (unlike the $\gamma 1$ chain) also avoids the wide spectrum of secondary biological properties, such as complement fixation and antibody-dependant cell-mediated cytotoxicity (ADCC), which may be undesirable properties.

The cDNA of the IFN $\alpha 2a$ can be obtained by reverse transcription and PCR, using RNA extracted from leukocytes which express IFN α . One such cell line, KG-1, can be obtained from the American Type Culture Collection (ATCC) in Rockville, Maryland, where it is held under number CCL 246. In the procedure used in making the hybrid of the invention, before the RNA extraction, the cells were challenged by Sendai virus to increase their transcription of interferons (Cantell, K. et al., *Methods in Enzymology*, 78A:29-38, Academic Press, 1981).

As mentioned above, IFN α is a collection of IFN species and each cell expresses several different IFN α subspecies at the same time. The DNA sequence homology among these species is so high that RT-PCR would probably amplify a group of them instead a specific one. To obtain specifically the IFN $\alpha 2a$ cDNA, the PCR primers were designed so that the last nucleotides of the two primers ended at positions where the amino acids coded are unique for IFN $\alpha 2a$. These are position S22 and 161, respectively (See Zoon, K.C. *Interferon*, 9:1-12, 1987).

By using an overlapping PCR technique (Daugherty, B.L. et al., *Nucleic Acids Res.* 19:2471-6, 1991), one can easily ligate two gene segments at any site as desired. However, one drawback of PCR amplification is the relatively high mutation rate (Saiki, R.K. et al., *Science*, 239:487, 1988). Thus, DNA sequencing was also done to check every DNA segment obtained through PCR for lack of

mutation. Sequencing can be tedious and time consuming when the size of the segment is over 1kb, as is the full length IFN α -Fc cDNA. However, a restriction endonuclease site, BamH I, can be incorporated into the linker nucleotide sequence without changing its amino acid sequence. This site is located between the nucleotide numbers 15 and 16 in SEQ ID NO:1.

5 The two gene segments from PCR can be separately cloned into cloning vectors. This makes the DNA sequencing easier and quicker since both segments are only a few hundred base pairs in length. Once the clones with the correct DNA sequences are identified, the two gene segments can be linked together through the BamH I site. No second round overlapping PCR and subsequent DNA sequencing of the full length segment are required.

10 There are several ways to express the recombinant protein *in vitro*, including in *E. coli*, baculovirus, yeast, mammalian cells or other expression systems. The prokaryotic system, *E. coli*, is not able to do post-translational modification, such as glycosylation. But this is probably not a serious problem for the IFN α -Fc hybrid since the native IFN α and immunoglobulin γ 4 molecule are not heavily glycosylated. Further, it has been reported that recombinant IFN α without any glycosylation retained
15 its biological activity (Baron, E. and Narula, S., *Bio/technology*, 10:179-190, 1990). However, the purification of recombinant protein from the *E. coli* lysate can be difficult. The foreign proteins expressed by *E. coli* often aggregate and form insoluble inclusion bodies. Thus, solubilization and subsequent refolding of the inclusion bodies is usually required (Schein, C.H. and Notetborn, H.M., *Bio/technology*, 6:291-294, 1988; Wilkinson, D.L. and Harrison, R.G., *Bio/technology*, 9:443-448, 1991).

20 The yeast expression system *Pichia Pastoris* (Invitrogen, San Diego, CA) overcomes some of the problems encountered when using the bacterial system. It usually gives a high yield and has the ability to do various post-translational modifications. The expressed foreign protein can be secreted

into the culture supernatant where not many other proteins reside, making protein purification and process scale-up much easier. This system was tried first to express either the IFN α -Fc hybrid or the wild type IFN α 2a. Unfortunately the IFN α -Fc secreted was found to be partially degraded on SDS-PAGE, whereas the IFN α 2a alone was not. The degradation was believed to be caused by the protease activities present in the yeast expression system, as reported by Scorer, C.A. et al., *Gene*, 136:111-9, 1993. The relatively weak spot in the hinge region is the possible target for the proteases.

A mammalian cell expression system for the IFN α -Fc hybrid was also tried. The mammalian expression vector, pCDNA3 (Invitrogen, San Diego, CA) which contains a CMV promoter and a NEO resistance gene, was employed. The host cells, NSO cells, were transfected by the pCDNA3/IFN α -Fc expression vector using the electroporation method. The cells were selected by G418 at a concentration of 0.8 mg/ml. The IFN α -Fc expressing clones were identified by ELISA. The hybrid was successfully expressed in this system and there was no degradation.

There are several advantages to this mammalian expression system. First, the recombinant protein is secreted into the culture supernatant and there is no aggregation, thereby simplifying purification. One chromatography step using a protein A column yields a purified IFN α -Fc protein. Also, the protein produced in this system has a glycosylation pattern very similar to the natural molecules since it is expressed by mammalian cells. Further, a native IFN α 2a signal peptide sequence is included in the expression vector. Therefore the protein secreted from the cells has an authentic N-terminal, whereas in the *E. coli* or yeast expression systems there either is no signal peptide or a non-IFN α signal peptide is used. Either way, it will bring in additional artificial amino acid residue(s) at the N-terminal end of the recombinant IFN α -Fc.

As mentioned above, the purification of the IFN α -Fc recombinant protein from the culture

supernatant is relatively straightforward. The protein with a purity of more than 90%, as judged by SDS-PAGE, can be easily obtained by one step of affinity chromatography with a protein A column.

There are several assay methods available for the measuring of the IFN α bioactivity. Using an antiviral assay, it was demonstrated that the hybrid of SEQ ID NO:7 had a specific activity about 5 to 10 fold higher than a related IFN α -Fc hybrid, in which the linker molecule had the sequence Gly Gly Ser Gly Gly Ser (SEQ ID NO:2), and the Fc portion of the hybrid was derived from human IgG1 rather than IgG4. Nevertheless, although the biological activity of the hybrid shown in SEQ ID NO:7 was improved substantially over the similar hybrid, it was still lower than that of the native IFN α . However, it is expected that this hybrid will have a longer half-life *in vivo*, than the native IFN α . This expectation is based on results demonstrating that the related IFN α hybrid with the linker sequence shown in SEQ ID NO:2 and an IgG1 Fc portion showed a much longer half-life, in a pharmacokinetic study in a mouse model, than did the native IFN α .

Because the hybrid of SEQ ID NO:7 is expected to have a longer half-life *in vivo* than native IFN α , even though its specific activity is lower, this novel hybrid is expected to be preferred to the native IFN α for clinical use. This is because, as a result of the longer half-life, the Cxt (the area under the concentration vs. time curve) would be up to several hundred times greater than for the native IFN α . This means that at the equivalent molar dosage of the native IFN α and the hybrid, the latter would provide a several hundred fold increased exposure to IFN α , resulting in vastly increased efficacy at the same dosage, and less frequent administration.

In measuring specific activity, molar dosage is preferred instead of expressing activity as units per mass of protein. This is because interferons function through the binding to their specific receptors, which is directly related to the number of molecules present. Also, the molecular weight

of the IFN α -Fc γ 4, 110 Kd, is more than five-fold larger than that of the wild type IFN α 2a, which is 20kd. Taking this into consideration, measuring activity in units/ μ mol instead of the units/mg provides a better comparison of activity specificity.

Example I: Cloning human IFN α cDNA and constructing the IFN α -Fc expression vector

5 6x10⁶ KG-1 cells (ATCC 246) were incubated with 200 units of Sendai virus at 37°C overnight. The cells were harvested and washed with PBS throughly. The total RNA was extracted by using the RNA-ZOL RNA isolation kit (BIOTEX, Houston, TX) following the procedure provided by the manufacturer. The first-strand cDNA was synthesized by reverse transcription using AMV reverse transcriptase with oligo(dT) as 3' primer in 50mM Tris-HCl (pH 8.3), 60mMKCl, and 6mM MgCl₂,
10 incubated at 42°C for 1 hour. The reaction mixture was used directly as the template for PCR to amplify IFN α cDNA. The 5' primer for PCR contained a Hind III site and the coding sequence for the first 21 amino acids from the IFN α 2a leader peptide (SEQ ID NO:3). The 3' primer contained the sequence coding for part of the linker (SEQ ID NO:1) and the last five amino acids of the IFN α 2a, and a BamH I site integrated in the linker sequence (SEQ ID NO:4). The PCR buffer contained 50mM KCl,
15 10mMTris-Hcl (pH8.3), 1.5mM MgCl₂, 0.01% gelatin, 0.1 mmol each of dNTP, 0.5 μ mol of each primers, 5 μ l RT reaction mixture, and 1 unit of Taq DNA polymerase in a total of 50 μ l volume. The PCR condition was 94°C (1 min), 55°C (2 min), and 72°C (2 min) for 40 cycles on a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT).

 The cDNA of the human immunoglobulin γ 4 Fc was obtained by reverse transcription and PCR
20 performed the same way as described above. The RNA was extracted from the human tonsil B cells. The 5' primer had the sequence shown in SEQ ID NO:5. The 3' primer had the sequence shown in SEQ ID NO:6.

The two PCR amplified DNA segments were cloned into pUC18 vectors at sites Hind III/BamH I or sites BamH I/EcoR I respectively. After their DNA sequences were confirmed by DNA sequencing using the kit from USB (Cleveland, Ohio), the two segments were ligated together through the BamH I site by a second round cloning. The full length IFN α -Fc cDNA was then inserted into a mammalian expression vector pCDNA3 (Invitrogen, San Diego, CA) through the Hind III and EcoR I sites.

Example 2: Expressing IFN α -Fc in mammalian cells

10⁷ NSO cells were mixed with 10 μ g linearized pCDNA3/IFN α -Fc plasmid in 0.8 ml PBS and kept on ice for 5 min. Electroporation was performed at 200v, 960 μ F using Gene Pulser (BioRad, Hercules, CA). The cells were then put back on ice for 20 minutes and transferred to a 100mm tissue culture plate in 10ml DMEM supplied with 2% FCS. After incubation at 37°C for two days, the cells were washed and resuspended in the same medium. 0.6 mg/ml G418 was added to start the selection. The cells were plated out in eight 96-well micro plates and incubated at 37°C. Colonies appeared in one week and they were ready for screening in two weeks. The supernatants from each well with a single colony growing were collected. The IFN α -Fc in the supernatant was quantitatively determined by an ELISA assay employing goat anti-human IgG and anti-human Fc conjugated with horseradish peroxidase. The clones with higher ELISA readings and smaller colony size were selected for subcloning. These colonies were transferred to a 24-well plate and supplied with a medium containing G418. The clone with the highest secretion level was expanded and adapted to grow in a spinner. For large scale preparation, the culture supernatant was collected and passed through a protein A agarose column equilibrated by PBS. The protein bound to the protein A was eluted by 50 mM citric acid (pH 3.0) and concentrated by lyophilization.

Example 3: Characterization of the IFN α -Fc hybrid.

The purity of the recombinant protein isolated from NSO culture medium was examined by SDS-PAGE and Western blot. Only one protein band was visible on the blotted membrane stained by ponceau s for total proteins, showing a homogeneity of the protein preparation. The apparent molecular weight of this protein is about 55kd under reducing conditions and 110kd under non-reducing conditions, which is exactly the predicted size for the IFN α -Fc hybrid. The doubling of its apparent molecular weight under non-reducing conditions suggests that the hybrid is in a dimeric form. The recombinant protein can be recognized by both anti-Fc and anti-IFN α antibodies, confirming that it consists of two moieties, the IFN α and the Fc fragment.

The bioactivity assay for the IFN α -Fc was an antiviral assay. Specifically, the assay method used was a modification of the protocol described by Robert M. Friedman et al (Measurement of antiviral activity induced by interferons α , β , and γ , *Current Protocols in Immunology*, 1994, pp. 6.9.1-6.9.8). Briefly, human lung carcinoma cells (A549, ATCC#CCL 185) were seeded in 96-well plates at a density of 40,000 cells/well and incubated at 37°C for 24 hours. 1:2 serially diluted IFN α -Fc hybrid or native IFN α (NIH# Gxa01-901-535) were added and incubated at 37°C for 24 hours. Every sample was done in triplicate. The culture medium was replaced with a fresh one containing encephalomyocarditis virus (ATCC #VR 129B) at a concentration of about 0.1 MOI/cell and incubated at 37°C for a further 48 hours. The dead cells were washed away by pipetting up and down vigorously with PBS. The attached cells were fixed by 2% formaldehyde and stained by giemsa stain. The plates were rinsed with tap water and allowed to dry. The stained cells were dissolved by methanol and the samples were read spectrophotometrically at 595nm. The antiviral activity of IFN α -Fc hybrid was calculated by comparing it with the IFN α standard, and was found to be about 30 to 60% of the activity of the IFN α standard.

It should be understood that the terms and expressions used herein are exemplary only and not limiting, and that the scope of the invention is defined only in the claims which follow, and includes all equivalents of the subject matter of those claims.

15

(A) Length: 81 nucleic acids
 (B) Type: nucleic acid
 (C) Strandedness: single stranded
 (D) Topology: linear
 (xi) Sequence Description: SEQ ID NO:3:

CATAAGCTTC ATCTACAATG GCCTTGACCT TTGCTTTACT 40
 GGTGGCCCTC CTGGTGCTCA GCTGCAAGTC AAGCTGCTCT G 81

(2) Information for SEQ ID NO:4:
 (i) Sequence Characteristics:
 (A) Length: 40 nucleic acids
 (B) Type: nucleic acid
 (C) Strandedness: single stranded
 (D) Topology: linear
 (xi) Sequence Description: SEQ ID NO:4:

CTCTGCGGAT CCACCTGAGC CACCTTCCTT ACTTCTTAAA 40

(2) Information for SEQ ID NO:5:
 (i) Sequence Characteristics:
 (A) Length: 58 nucleic acids
 (B) Type: nucleic acid
 (C) Strandedness: single stranded
 (D) Topology: linear
 (xi) Sequence Description: SEQ ID NO:5:

AATGGATCCG GTGGAGGCGG AAGCGGCGGT GGAGGATCAG 40
 AGTCCAAATA TGGTCCCC 58

(2) Information for SEQ ID NO:6:
 (i) Sequence Characteristics:
 (A) Length: 42 nucleic acids
 (B) Type: nucleic acid
 (C) Strandedness: double stranded
 (D) Topology: linear
 (xi) Sequence Description: SEQ ID NO:6:

ATCGAATTCT ATTTACCCAG AGACAGGGAG AGGCTCTTCT GT 42

(2) Information for SEQ ID NO:7:
 (i) Sequence Characteristics:
 (A) Length: 1302 nucleic acids
 (B) Type: nucleic acid
 (C) Strandedness: double stranded
 (D) Topology: linear
 (xi) Sequence Description: SEQ ID NO:7:

ATG GCC TTG ACC TTT GCT TTA CTG GTG GCC CTC CTG GTG 39
 Met Ala Leu Thr Phe Ala Leu Leu Val Ala Leu Leu Val
 1 5 10

CTC AGC TGC AAG TCA AGC TGC TCT CTG GGC TGT GAT CTG 78
 Leu Ser Cys Lys Ser Ser Cys Ser Leu Gly Cys Asp Leu
 15 20 25

CCT CAA ACC CAC AGC CTG GGT AGC AGG AGG ACC TTG ATG 117
 Pro Gln Thr His Ser Leu Gly Ser Arg Arg Thr Leu Met
 30 35

16

	CTC	CTG	GCA	CAG	ATG	AGG	AAA	ATC	TCT	CTT	TTC	TCC	TGC	156
	Leu	Leu	Ala	Gln	Met	Arg	Lys	Ile	Ser	Leu	Phe	Ser	Cys	
	40					45					50			
5	TTG	AAG	GAC	AGA	CAT	GAC	TTT	GGA	TTT	CCC	CAG	GAG	GAG	195
	Leu	Lys	Asp	Arg	His	Asp	Phe	Gly	Phe	Pro	Gln	Glu	Glu	
			55					60					65	
10	TTT	GGC	AAC	CAG	TTC	CAA	AAG	GCT	GAA	ACC	ATC	CCT	GTC	234
	Phe	Gly	Asn	Gln	Phe	Gln	Lys	Ala	Glu	Thr	Ile	Phe	Val	
					70					75				
15	CTC	CAT	GAG	ATG	ATC	CAG	CAG	ATC	TTC	AAT	CTC	TTC	AGC	273
	Leu	His	Glu	Met	Ile	Glu	Glu	Ile	Phe	Asn	Leu	Phe	Ser	
		80					85					90		
20	ACA	AAG	GAC	TCA	TCT	GCT	GCT	TGG	GAT	GAG	ACC	CTC	CTA	312
	Thr	Lys	Asp	Ser	Ser	Ala	Ala	Trp	Asp	Glu	Thr	Leu	Leu	
				95					100					
25	GAC	AAA	TTC	TAC	ACT	GAA	CTC	TAC	CAG	CAG	CTG	AAT	GAC	351
	Asp	Lys	Phe	Tyr	Thr	Glu	Leu	Tyr	Gln	Gln	Leu	Asn	Asp	
	105					110					115			
30	CTG	GAA	GCC	TGT	GTG	ATA	CAG	GGG	GTG	GGG	GTG	ACA	GAG	390
	Leu	Glu	Ala	Cys	Val	Ile	Gln	Gly	Val	Gly	Val	Thr	Glu	
			120					125					130	
35	ACT	CCC	CTG	ATG	AAG	GAG	GAC	TCC	ATT	CTG	GCT	GTG	AGG	429
	Thr	Pro	Leu	Met	Lys	Glu	Asp	Ser	Ile	Leu	Ala	Val	Arg	
					135					140				
40	AAA	TAC	TTC	CAA	AGA	ATC	ACT	CTC	TAT	CTG	AAA	GAG	AAG	468
	Lys	Tyr	Phe	Gln	Arg	Ile	Thr	Leu	Tyr	Leu	Lys	Glu	Lys	
		145					150					155		
45	AAA	TAC	AGC	CCT	TGT	GCC	TGG	GAG	GTT	GTC	AGA	GCA	GAA	507
	Lys	Tyr	Ser	Phe	Cys	Ala	Trp	Glu	Val	Val	Arg	Ala	Glu	
				160					165					
50	ATC	ATG	AGA	TCT	TTT	TCT	TTG	TCA	ACA	AAC	TTG	CAA	GAA	546
	Ile	Met	Arg	Ser	Phe	Ser	Leu	Ser	Thr	Asn	Leu	Gln	Glu	
	170					175					180			
55	AGT	TTA	AGA	AGT	AAG	GAA	GGT	GGC	TCA	GGT	GGA	TCC	GGT	585
	Ser	Leu	Arg	Ser	Lys	Glu	Gly	Gly	Ser	Gly	Gly	Ser	Gly	
			185					190					195	
60	GGA	GGC	GGA	AGC	GGC	GGT	GGA	GGA	TCA	GAG	TCC	AAA	TAT	624
	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Glu	Ser	Lys	Tyr	
					200					205				
65	GGT	CCC	CCG	TGC	CCA	TCA	TGC	CCA	GCA	CCT	GAG	TTC	CTG	663
	Gly	Pro	Pro	Cys	Pro	Ser	Cys	Pro	Ala	Pro	Glu	Phe	Leu	
		210					215					220		
70	GGG	GGA	CCA	TCA	GTC	TTC	CTG	TTC	CCC	CCA	AAA	CCC	AAG	702
	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	
				225					230					

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	GAC	ACT	CTC	ATG	ATC	TCC	CGG	ACC	CCT	GAG	GTC	ACG	TGC	741
	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	
	235					240					245			
5	GTG	GTG	GTG	GAC	GTG	AGC	CAG	GAA	GAC	CCC	GAG	GTC	CAG	780
	Val	Val	Val	Asp	Val	Ser	Gln	Glu	Asp	Pro	Glu	Val	Gln	
			250					255					260	
10	TTC	AAC	TGG	TAC	GTG	GAT	GGC	GTG	GAG	GTG	CAT	AAT	GCC	819
	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala	
					265					270				
15	AAG	ACA	AAG	CCG	CGG	GAG	GAG	CAG	TTC	AAC	AGC	ACG	TAC	858
	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Phe	Asn	Ser	Thr	Tyr	
		275					280					285		
20	CGT	GTG	GTC	AGC	GTC	CTC	ACC	GTC	CTG	CAC	CAG	GAC	TGG	897
	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	
				290					295					
	CTG	AAC	GGC	AAG	GAG	TAC	AAG	TGC	AAG	GTC	TCC	AAC	AAA	936
	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	
	300					305					310			
25	GGC	CTC	CCG	TCC	TCC	ATC	GAG	AAA	ACC	ATC	TCC	AAA	GCC	975
	Gly	Leu	Pro	Ser	Ser	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	
			315					320					325	
30	AAA	GGG	CAG	CCC	CGA	GAG	CCA	CAG	GTG	TAC	ACC	CTG	CCC	1014
	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	
					330					335				
35	CCA	TCC	CAG	GAG	GAG	ATG	ACC	AAG	AAC	CAG	GTC	AGC	CTG	1053
	Pro	Ser	Gln	Glu	Glu	Met	Thr	Lys	Asn	Gln	Val	Ser	Leu	
		340					345					350		
40	ACC	TGC	CTG	GTC	AAA	GGC	TTC	TAC	CCC	AGC	GAC	ATC	GCC	1092
	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	
				355					360					
	GTG	GAG	TGG	GAG	AGC	AAT	GGG	CAG	CCG	GAG	AAC	AAC	TAC	1131
	Val	Glu	Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	
	365					370					375			
45														
50	AAG	ACC	ACG	CCT	CCC	GTG	CTG	GAC	TCC	GAC	GGC	TCC	TTC	1170
	Lys	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	
			380					385					390	
	TTC	CTC	TAC	AGC	AGG	CTA	ACC	GTG	GAC	AAG	AGC	AGG	TGG	1209
	Phe	Lys	Tyr	Ser	Arg	Leu	Thr	Val	Asp	Lys	Ser	Arg	Trp	
					395					400				
55	CAG	GAG	GGG	AAT	GTC	TTC	TCA	TGC	TCC	GTG	ATG	CAT	GAG	1248
	Gln	Glu	Gly	Asn	Val	Phe	Ser	Cys	Ser	Val	Met	His	Glu	
		405					410					415		
60	GCT	CTG	CAC	AAC	CAC	TAC	ACA	CAG	AAG	AGC	CTC	TCC	CTG	1287
	Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	
				420					425					

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TCT CTG GGT AAA TAG 1302
Ser Leu Gly Lys
430

What Is Claimed Is:

1. A hybrid molecule comprising an interferon molecule joined at its C-terminal end through a peptide linker to the N-terminal end of the immunoglobulin Fc fragment, the peptide linker comprising the sequence Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly
5 Ser Gly Gly Gly Gly Ser (SEQ ID NO:1).
2. The hybrid molecule of claim 1 in which another interferon molecule is joined at its C-terminal end through the peptide linker to the N-terminal end of a chain of the immunoglobulin Fc fragment, thereby forming a homodimer.
3. The hybrid molecule of claim 2 in which the interferon molecule is IFN α 2a
10 or IFN α 2b.
4. The hybrid molecule of claim 2 in which the Fc fragment is a γ 4 chain Fc fragment.
5. A method of treating hepatitis, hairy cell leukemia, multiple myeloma, or other cancers or viral diseases, comprising administering the hybrid molecule of any of
15 claims 1 to 4.

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/21, 39/395, 39/44; C07K 14/56

US CL : 424/134.1; 435/69.7; 514/2, 12, 934; 530/351, 387.3; 536/23.52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/134.1; 435/69.7; 514/2, 12, 934; 530/351, 387.3; 536/23.52

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, EMBASE, BIOSIS, WORLD PATENT INDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,349,053 A (LANDOLFI) 20 September 1994, columns 4 and 13.	1-5
Y	WO 91/16353 A1 (CORVAS INTERNATIONAL N.V.) 31 October 1991, figure 6, sequence 9, pages 16-20.	1-5
Y	BARON, E. et al. From Cloning to a Commercial Realization: Human Alpha Interferon. Biotechnology. 1990. Vol. 10. No. 3. pages 197-190, see especially page 185.	1-5

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

14 FEBRUARY 1997

Date of mailing of the international search report

04 APR 1997

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(54) **Covalently linked polypeptide cell modulators.**

(57) Described is a new class of polypeptide cell modulators characterized by being composed of two covalently linked cell modulators in a linear polypeptide sequence. Such dual function polypeptides have new and particularly useful activities when the component polypeptide cell modulators are interferons, lymphokines or cytotoxins which act through different and specific cell receptors to initiate complementary biological activities.

EP 0 225 579 A2